PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Curtis R. Brandt *et al.*

Title: PHARMACOLOGICALLY ACTIVE ANTIVIRAL PEPTIDES AND METHODS OF THEIR USE

Appl. No.: 09/777,560

Filing Date: 02/06/2001

Examiner: Stacy B. Chen

Art Unit: 1648

DECLARATION UNDER 37 C.F.R. §1.132 OF DR. CURTIS R. BRANDT

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Examiner:

I, Dr. Curtis R. Brandt, state and declare that:

1. I am a citizen of the United States of America, residing at 416 Nottingham Rd., Stoughton, WI 53589.

2. I received my Bachelor of Science degree in Microbiology in 1975 from Washington State University. I received a Masters of Science degree in Microbiology in 1977 from Washington State University. In 1983, I received a Masters in Philosophy in Microbiology from Columbia University, New York. In 1984, I received my Ph.D. from Columbia University in Microbiology.

3. I am Professor of Ophthamalogy and Visual Sciences and Medical Microbiology and Immunology at the University of Wisconsin-Madison, and have conducted research into viruses, viral infections, and antiviral agents for 22 years.

4. I am a co-inventor of U.S. Application Serial No. 09/777,560 (hereinafter "the '560 Application").

5. I have reviewed the Office Action mailed 11/07/2005 (hereinafter "the Office Action"), the prior art cited therein, and the amended claims.

6. The claimed invention is directed to antiviral peptides having membrane transiting sequence motifs and compositions and methods of use of these peptides.

7. The '560 Application teaches that the claimed peptides are suitable for use as antiviral agents against a wide variety of viruses and presents in vitro and in vivo data regarding the activity of peptides of the invention with regard to herpes simplex virus. Based on the teachings of the '560 Application, I and my co-inventor Hermann Bultmann have used the peptides and methods as taught and claimed in pending claims 1-17 of the '560 Application to inhibit viral activity of HIV, influenza A and H5N1 avian influenza, human and bovine papillomavirus, and vaccinia virus.

8. In copending application 11/001,674 (U.S. Patent Publication No. US 2005/0203024, hereinafter "the '024 Application"), my co-inventor Hermann Bultmann and/or I performed or caused to be performed the experiments of Examples 10, 11, 12, and 13. These Examples demonstrate that peptides claimed in the '560 Application possess antiviral activity against HIV (Example 10), influenza A (Examples 11, 12, and 13), and H5N1 avian influenza virus (Example 12). In addition, in the (second) Example 13 that begins with paragraph 162, bTAT-9 (SEQ ID NO:10) and bTAT-9X (SEQ ID NO:11) are shown to selectively block viral entry of Herpes Simplex Virus hrR3 into Vero cells. The blockage of viral entry demonstrated for these peptides is consistent with that demonstrated by other claimed peptides in Example 7 of the '560 Application.

9. My co-inventor Hermann Bultmann and/or I performed or caused to be performed the experiments of Examples A – E described in the attached Appendix to this declaration. The results of these experiments show that claimed peptides, e.g., EB (SEQ ID NO:1), inhibit infection of HeLA cells by the pox family virus, vaccinia virus (Example A), and does so by reversibly binding to the virus (Example B) rather than by conferring resistance on the host cell (Example C). Claimed peptides bind to specific vaccinia proteins (Example D), but do not prevent attachment of the virus to the host cell (Example E).

10. My co-inventor Hermann Bultmann and/or I, and/or Paul Lambert of the University of Wisconsin and/or Michelle Ozbun of the University of New Mexico, performed or caused to be performed experiments demonstrating that claimed peptides inhibit human and bovine papillomavirus infection. Example F of the Appendix shows that peptides EB (SEQ ID NO: 1) and KLA inhibit HPV31 infection in cells. Example G shows that peptide EB and other peptides of the invention inhibit infection of cells by bovine papillomavirus type 1. Of

all the viruses we have tested, only adenovirus has shown resistance to peptides of the invention.

11. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both under Section 001 of Title 18 of the United State Code and that such willful false statements may jeopardize the validity of the application or any patent resulting therefrom.

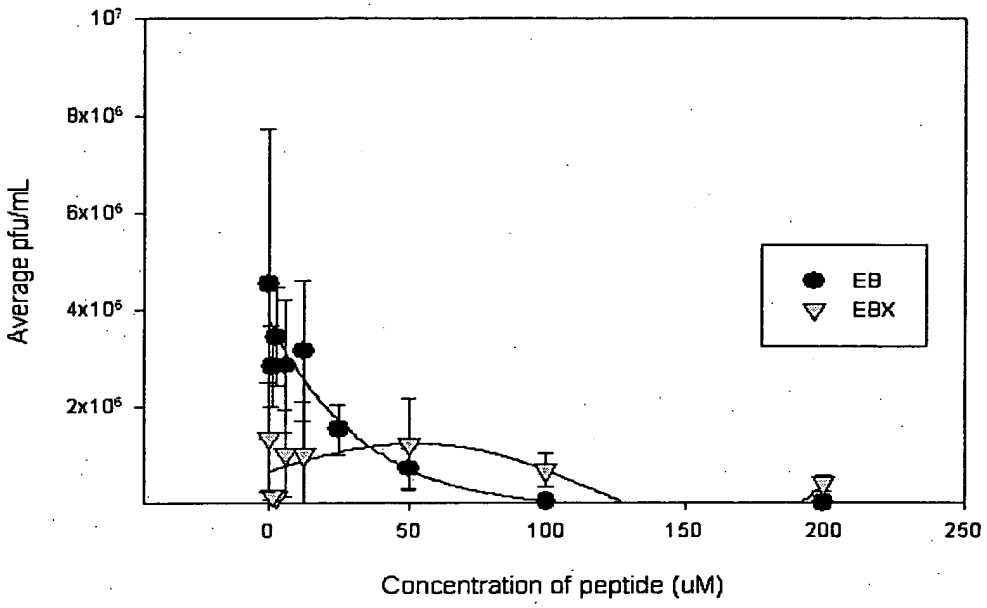
Date: May 5, 2006

Curtis R. Brandt Ph.D.

Dr. Curtis R. Brandt

APPENDIX

EXAMPLE A: EB inhibits vaccinia viral infection more effectively than EBX

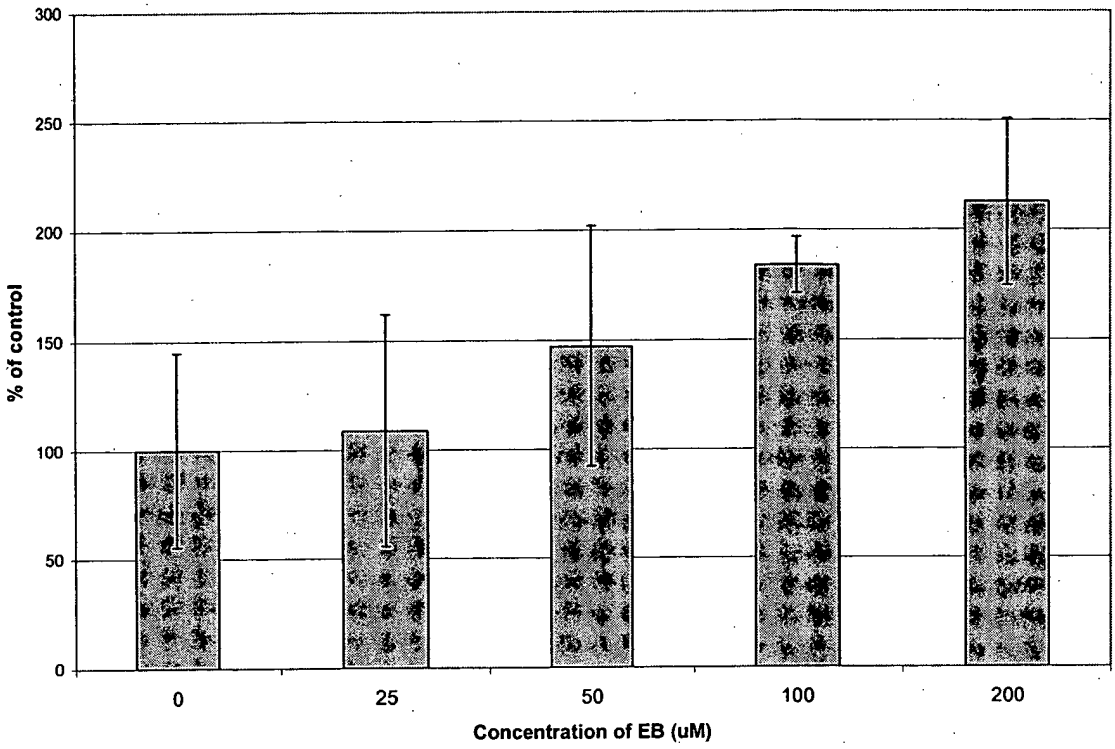


Comprehensive inhibition assay. Equal numbers of vaccinia virus were incubated for 1 hr @ 37°C in the presence of a given amount of peptide. Samples were then serially diluted into peptide-containing DMEM +2% FBS:CS, plated onto confluent HeLA cells in a 24-well plate, and incubated at 37°C until 90% CPE was observed. Plates were then frozen and thawed. The amount of virus present in each condition was measured by titering on confluent HeLA plates.

IC_{50} EB = 15 uM
IC_{50} EBX = 50 uM

Conclusion: EB is a more effective antiviral than EBX and the amino acid sequence of the peptide is important to its antiviral activity.

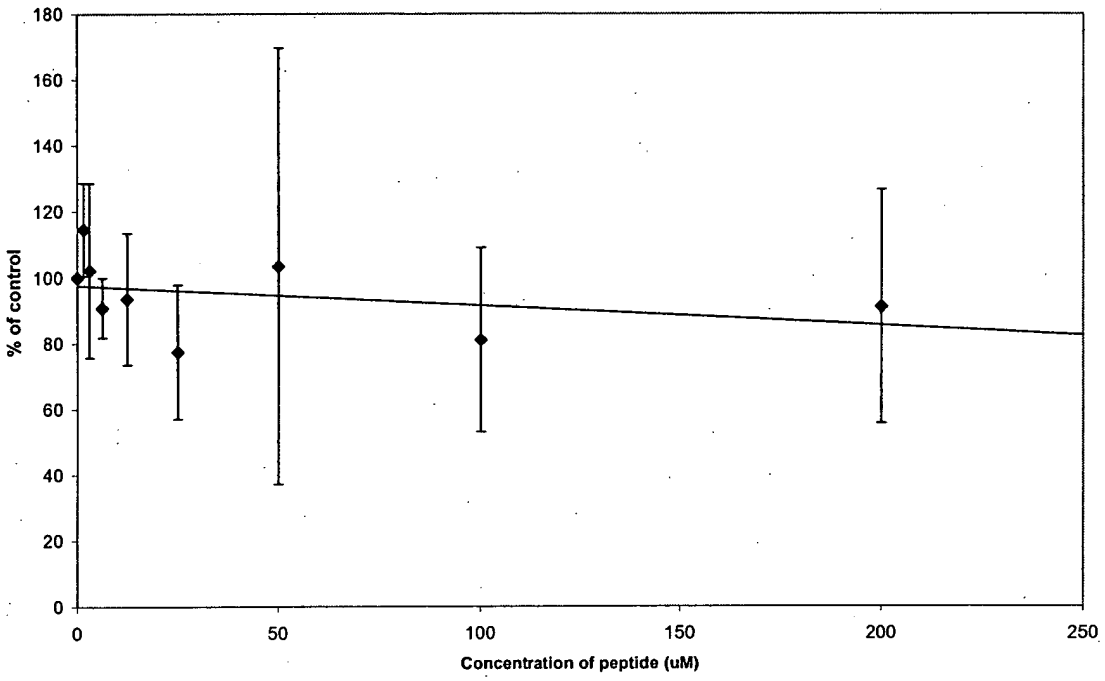
EXAMPLE B: EB is not viricidal



Dialysis restores vaccinia infectivity. A known amount of virus was incubated for 1 hr at 37°C with given amount of peptide. Samples were then transferred to Slide-A-Lyzer Dialysis Cassettes (Pierce, #66425) and dialyzed in 10 mM Tris, 0.1 M NaCl pH 7.4 as per the dialyis cassette instructions. After dialysis, samples were titered and the pfu's recovered were compared to the pfu's recovered from dialyzed virus that was not exposed to peptide.

Conclusion: Dialysis of peptide-treated vaccina can restore full viral infectivity. This suggests that EB reversibly binds to a target on the virus and that EB does not grossly alter virus structure.

EXAMPLE C: Pretreatment of HeLA cells with EB does not confer resistance to vaccinia infection



Confluent 6-well plates of HeLA cells were incubated with peptide for 30 minutes at 37°C. Plates were then washed 3x with media and incubated with virus for 1 hour at 37°C. Media was removed and replaced with a methylcellulose overlay. Plates were incubated 3 days before being fixed and stained. The number of plaques per well was counted and compared to the number of plaques present in wells not treated with peptide.

Conclusion: Pretreatment of cells followed by washout of the peptide does not confer resistance against vaccinia infection. Antiviral effects are due solely to interactions between the peptide and the virus.

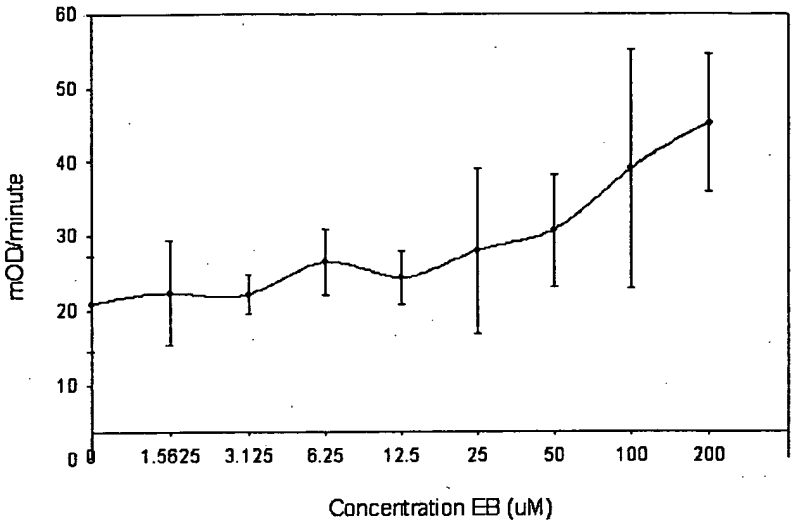
EXAMPLE D: EB binds specifically to vaccinia virus proteins.

Preliminary data suggest that EB must interact directly with vaccinia virus in order to exhibit antiviral effect. To determine if EB binds specifically to particular vaccinia virus proteins, a Far Western blot was performed on whole-vaccinia virus lysate under four conditions: 1:1 biotinylated EB (bEB) to EB, 1:2 bEB to EB, and bEBX. The banding patterns in the bEB, 1:1 bEB:EB, and 1:2 bEB:EB was comparable, with the intensity of the signal decreasing as the percentage of unlabeled EB increased.

Two of the most intense bands in the bEB blots, at 54 kDa and 58 kDa, were sequenced by mass spectrometry at the University of Wisconsin-Madison Biotechnology Center. They were identified as proteins p4a and p4b, major components of the viral core. The third intense band (23 kDa) was not present in high enough concentrations to process, although based on its size possible identities include F9, or the envelope proteins H2, H5 and A17.

Conclusions: Probing with bEB or bEBX resulted in different banding patterns, suggesting that they have different binding specificity. Unbiotinylated EB competed with bEB for binding in a concentration-dependent manner, suggesting that binding is specific.

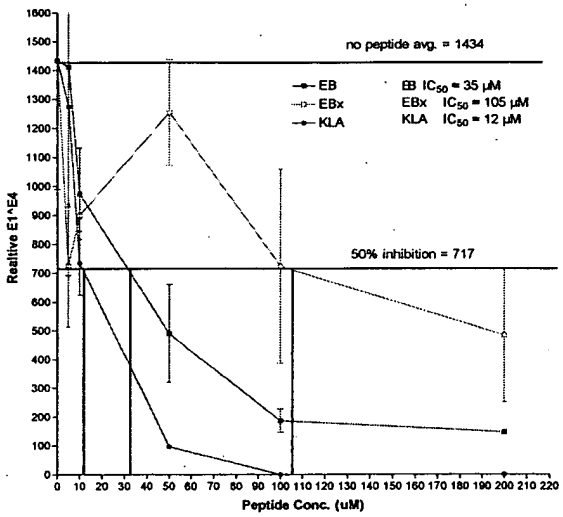
EXAMPLE E: EB does not inhibit vaccinia virus attachment



Confluent HeLa cells in 96-well plates were infected with peptide-treated or untreated vMJ343, a recombinant virus containing functional -galactosidase (gift of B. Moss), at 4°C for 1 hr before unattached virus was removed by washing. Samples were lysed and developed with chlorophenol red-beta-D-galactopyranoside, a -galactosidase substrate. Virus binding as measured by -galactosidase activity increased in direct proportion to the amount of EB present.

Conclusions: This suggests that vaccinia virus attachment is not inhibited by EB. The apparent increase in virus attachment after treatment with concentrations of EB higher than 25 uM is believed to be due to peptide-induced aggregation. As the IC_{50} of the virus, 15 uM, is less than this value, it is unlikely that aggregation is contributing to the antiviral effect of the peptide.

EXAMPLE F: Inhibition of HPV31 infection by selected peptides



HPV31b virions (3 x 10^6 DNA containing particles or VGE) were incubated with the indicated peptides and the HaCaT cells were exposed to HPV31b at 10 /cell. The data are expressed as levels of HPV31b E1^E4 transcripts relative to the control, which was not treated with peptide (0 point on the x-axis, average of 1434 copies of E1^E4 targets per qPCR analysis). Error bars reflect the standard error of the mean for triplicate qPCR reactions. Note the ability of KLA and EB peptides to inhibit infection at IC_{50} of 12 and 35 uM, respectively. Scrambled EB peptide (EBx) shows an IC_{50} greater than 100 uM.

EXAMPLE G: Inhibition of Bovine Papillomavirus type 1 Infection by Selected Peptides Scored in the Mouse C127 Cell Transformation Assay

Peptide	IC_{50} (μM)
EB	34, 20‡
EBX	>200
bHOM D #1885	7.9,>2‡
bHOM D #2799	11
αHOM D	6.5, 7.8
bHOM FF	>200
HOM P50P69	>200
bHOMX	>200
bHOM-9	160,>200‡
bTAT-9	>200
TAT-cd	>200
bTAT-9X MIX	110
bKLA	nd‡

‡ In cases of EB, bHOMD #1885, and bHOM-9, shown are average IC_{50} values from two different experiments, each one done in triplicate.

Methodology: Mouse C127 cells, seeded at 1 x 10^5 cells/ 6 cm dish 24 hours prior to infection, were exposed to virus (1:100 dilution of stock virus that had or had not been preincubated for one hour at 37°C with serial dilutions of peptide in a final volume of 100 ul) in 1 mL of serum free medium for 4 hours at 37°C. Media was then aspirated and cells washed 2X with PBS. Fresh media was added and changed every three days. On day 18+ media was removed and foci stained with methylene blue and counted.